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Identification and Functional Characterization of CLCN1 Mutations Found in Nondystrophic Myotonia Patients

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Abstract

Mutations in the gene coding for the skeletal muscle Cl⁻ channel (CLCN1) lead to dominant or recessive myotonia. Here, we identified and characterized CLCN1 mutations in Costa Rican patients, who had been clinically diagnosed with myotonic dystrophy type 1 but who were negative for DM1 mutations. CLCN1 mutations c.501C>G, p.F167L and c.1235A>C, p.Q412P appeared to have recessive inheritance but patients had atypical clinical phenotypes; c.313C>T, p.R105C was found in combination with c.501C>G, p.F167L in an apparently recessive family and the c.461A>G, p.Q154R variant was associated with a less clear clinical picture. In *Xenopus* oocytes, none of the mutations exhibited alterations of fast or slow gating parameters or single channel conductance, and mutations p.R105C, p.Q154R, and p.F167L were indistinguishable from wild-type (WT). p.Q412P displayed a dramatically reduced current density, surface expression and exerted no dominant negative effect in the context of the homodimeric channel. Fluorescently tagged constructs revealed that p.Q412P is expressed inefficiently. Our study confirms p.F167L and p.R105C as myotonia mutations in the Costa Rican population, whereas p.Q154R may be a benign variant. p.Q412P most likely induces a severe folding defect, explaining the lack of dominance in patients and expression systems, but has WT properties once expressed in the plasma membrane.

Introduction

The nondystrophic myotonias (NDM) are a group of skeletal muscle hereditary disorders caused by the alteration of the function of voltage-gated ion channels. These diseases are mainly characterized by electrical and/or clinical myotonia (prolongation of muscle relaxation time after a voluntary contraction or external mechanical stimulation), muscle stiffness, occasional muscle hypertrophy, but lack of progressive muscle weakness/wasting and dystrophic histopathology [Thomsen, 1876; Becker, 1977; Lossin and George, 2008; Planells-Cases and Jentsch, 2009]. NDM are divided into two groups depending on which ion channel is mutated. Although myotonia congenita (MC) is associated with chloride channel mutations, paramyotonia congenita, hyperkalemic periodic paralysis with myotonia, myotonia fluctuans, myotonia permanens, and acetazolamide-sensitive myotonia are associated with sodium channel mutations [Trip et al., 2009; Jurkat-Rott et al., 2010; Matthews et al., 2010].

MC is a muscle disease that can be inherited as autosomal dominant (Thomsen disease; MIM# 160800) or recessive (Becker generalized myotonia; MIM# 255700) and is the most common form of NDM. This disease is characterized by hyperexcitability of the muscle fiber, muscle stiffness, and hypertrophy [Thomsen, 1876; Becker, 1977]. Becker type myotonia is more common and usually more severe [Becker, 1977]. The two forms of MC differ clinically by the age of onset, spreading of myotonia, and a typical transient muscular weakness present only in the recessive trait [Koch et al., 1992; Koch et al., 1993].

Onset of MC occurs during the first or second decade of life (usually earlier in Thomsen disease) and muscle stiffness can affect every skeletal muscle in the body [Lossin and George, 2008]. Myotonia in MC is clinically highly variable, ranging from myotonic discharges detected only by electromyography (EMG) to disabling muscle stiffness at an early age [Thomsen, 1876; Becker, 1977]. The intensity of myotonia can diminish with repeated movements, a phenomenon known as warm-up.

MC is caused by mutations in the CLCN1 gene on chromosome 7q35 encoding the skeletal muscle chloride channel (ClC-1) [Koch et al., 1992; George et al., 1993; Lorenz et al., 1994]. Expression of ClC-1 is almost exclusively restricted to skeletal muscle and accounts for its high resting chloride conductance. Each monomer of the channel is composed of 18 intramembrane helices, some of which do not span the membrane entirely, and a large cytoplasmic carboxy-terminus, which contains two cystathionine beta synthase domains. The channel functions as a homodimer, consisting of two identical subunits each containing a proper ion-conducting pore [Zifarelli and Pusch, 2007; Jentsch, 2008]. To date, more than 200 myotonia-related CLCN1 mutations are known, as reported in the Human Gene Mutation Database (<http://www.hgmd.cf.ac.uk/ac/index.php>). Nonsense and missense mutations lead to a loss of channel function causing recessive Becker generalized myotonia. These gene defects could induce impaired trafficking to the plasma membrane, altered voltage dependence, and reduced conductance [Planells-Cases and Jentsch, 2009]. The dominant Thomsen disease can be explained by dominant negative effects that may result from disturbed trafficking or early degradation of wild-type (WT)/mutant heteromers, or from changed biophysical properties of these heteromers [Steinmeyer et al., 1994; Pusch et al., 1995], and is mostly caused by missense mutations. However, also a few nonsense mutations have been found in patients affected by Thomsen disease [Colding-Jorgensen, 2005]. Insertions and deletions of single or

multiple nucleotides have also been described causing MC. However, a clear distinction between dominant and recessive mutations is not always possible, as several mutations have been identified in both recessive and dominant traits, making difficult the understanding of the relationship between genotype and phenotype [Kubisch et al., 1998; Plassart-Schiess et al., 1998; Colding-Jorgensen, 2005].

In this study, we report clinical, molecular, and electrophysiological data from several Costa Rican MC families, in which the probands carry new or already reported mutations, and unclear phenotypes.

Materials and Methods

Patients

The study involved 18 individuals from four apparently unrelated families previously diagnosed with myotonic dystrophy type 1 (DM1). However, molecular diagnosis for DM1 was negative. The four probands (one for each family) were clinically affected and presented symptoms suggesting a myotonic disorder. Clinical re-evaluation (including EMG in proband F1-CR3; Table 1) was carried out in two probands and the daughter of one of the probands (including EMG for F4-CR17; Table 1). A deep review of clinical records was carried out in the four probands. Some of the probands' relatives (14 in total) were also included in the study, and most of them remained asymptomatic at the moment of sampling. Signed informed consent was obtained for all subjects in accordance with the ethical protocols approved by the Ethical Scientific Committee of the University of Costa Rica.

Clinical Diagnosis

Clinical data were obtained from physical and EMG tests or after reviewing the clinical records. A complete neurological evaluation in the clinically re-evaluated cases focused on muscles, analyzing the strength, the presence of the myotonic phenomenon before the muscular percussion and in the relaxation phase after a voluntary contraction. The muscular strength was recorded according to the Medical Research Council scale. Data on the motor neuroconduction study, including distal motor latency, motor nerve conduction velocities, F-M latencies, and extent of the action potential of the median, ulnar, tibial, and peroneal nerves, were collected. We also obtained information from measured sensory nerve conduction velocities and sensory nerve action potentials of the right median, ulnar and sural nerves. All tests were performed at skin temperature of 31°C. We collected the same clinical information for all five patients included in this study.

Genetic Analysis

Genomic DNA was extracted from peripheral blood leucocytes using the proteinase K/phenol-chloroform approach. The entire coding sequence of the CLCN1 gene (RefSeq NM 000083.2) was amplified by PCR using intronic primers following previously published conditions [Lehmann-Horn et al., 1995] with minor modifications [Morales et al., 2008]. The PCR products of the 23 CLCN1 exons were analyzed using a previously described SSCP assay [Orita et al., 1989; Morales et al., 2008]. Exons showing aberrant conformers were sequenced after purification of the PCR products (Qiagen, Valencia, CA, USA). Bidirectional DNA sequencing was done according to

Big Dye Terminator Kit version 3.1 (Applied Biosystems, Waltham, MA, USA) and run in the ABI PRISM 377 sequencer. Sequences were analyzed with 4Peaks program (mekentosj.com).

The mutations identified were physically confirmed in the genomic DNA of the probands by RFLP-PCR with an appropriate restriction enzyme following the manufacturer's instructions (Supp. Table S1). For novel mutations, RFLP-PCR was conducted in 100 healthy unrelated individuals or with a disease different to NDM. Genotyping of the samples was based on the band pattern obtained after electrophoresis in agarose gels. Once the mutation was confirmed in the proband, we analyzed their relatives in order to identify the genetic change in other family members. Details on the genotyping of the samples can be requested. DNA mutation numbering is based on cDNA sequence.

Molecular Biology

Mutants c.313C>T, p.R105C; c.461A>G, p.Q154R; c.501C>G, p.F167L; and c.1235A>C, p.Q412P were constructed in human CIC-1 in the pTLN vector [Estevez et al., 2003] by recombinant PCR as described previously [Accardi and Pusch, 2003]. For expression in *Xenopus laevis* oocytes, cRNA of CIC-1 was transcribed by the mMessage mMachine SP6 kit (Ambion - Life Technologies, Milan, Italy) after linearization with Mlu I. For transfection, constructs were inserted in the pFrog vector (Picollo and Pusch, 2005). In this vector, mCherry was fused C-terminally to WT CIC-1 and p.Q412P using a 4 amino acid linker [Jeworutzki et al., 2012].

Oocyte Measurements

CIC-1-cRNA was injected in defolliculated *Xenopus* oocytes, which were incubated at 18°C in the maintaining solution containing (in mM): 90 NaCl, 2 KCl, 1 MgCl₂, 1 CaCl₂, and 10 Hepes (pH 7.5). Currents were measured using the two-electrode voltage-clamp technique and the inside-out configuration of the patch-clamp technique as described previously [Hamill et al., 1981; Estevez et al., 2003].

The voltage-clamp measurements were performed at room temperature using the custom acquisition program GePulse (available at <http://users.ge.ibf.cnr.it/pusch/programs-mik.htm>).

In the voltage-clamp measurements, the membrane was kept at a holding potential corresponding to the resting membrane potential (~−30 mV). To evaluate CIC-1 currents at different voltages, a stimulation protocol was applied using a prepulse to +60 mV for 20 msec followed by steps to various test values (from −140 to +80 mV in 20-mV increments) for 100 msec and a final tail pulse to −100 mV.

The composition of the extracellular solutions were: 100 mM NaCl, 10 mM HEPES-Na, 5 mM MgSO₄; endogenous currents were estimated by using a solution containing 100 mM NaI, 5 mM MgSO₄, and 10 mM Hepes (pH 7.3) that inhibits CIC-1 channels [Pusch et al., 2001].

Patch-clamp inside-out configuration measurements were performed at 18 ± 1°C using the following “tail protocol” from a 0 mV holding potential: a 200 msec test pulse to a variable potential ranging from −100 to +160 mV was followed by a repolarization

to -160 mV for 100 msec. To study in isolation slow gating transitions, we applied 200 msec test pulses to various voltages, followed by a 200 μ sec pulse to $+160$ mV to fully activate the fast gating before the -160 mV repolarization as described in detail earlier [Accardi et al., 2001].

The composition of the extracellular solution was 100 mM NMDG-Cl, 10 mM NMDG-HEPES, 2 mM $MgCl_2$, 1 mM EGTA, pH 7.3. We pulled pipettes from aluminum silicate glass, and the (extracellular) pipette solution contained 100 mM NMDG-Cl, 10 mM NMDG-HEPES, 5 mM $MgSO_4$, pH 7.3.

Open probabilities were derived from the tail currents by fitting a modified Boltzmann function [Pusch et al., 1995]:

$$I(V) = I_{\max} * (P_{\min} + (1 - P_{\min}) / (1 + \exp((V_{1/2} - V) / k)))$$

where $I_{\max}$ is the (fitted) maximal current, $P_{\min}$ is the residual open probability at negative voltages, $V_{1/2}$ is the midpoint of the voltage of open probability, and k the slope factor of the voltage dependence of the open probability.

Data analysis was performed using self-written software (Visual C++, Microsoft) and the SigmaPlot program (Systat Software, Inc., San Jose, CA). Estimates for the single-channel conductance were obtained by nonstationary noise analysis as described [Pusch et al., 1994; Saviane et al., 1999]. For noise analysis, ~ 100 identical pulses to -140 mV were applied, and the mean response, I , was calculated. The variance, σ^2 , was calculated from the averaged squared difference of consecutive traces. Background variance at 0 mV was subtracted. The variance-mean plot was fitted by $\sigma^2 = iI - I^2/N$, with the single-channel current, i , and the number of channels, N , as free parameters.

Surface Expression

To assay the surface membrane expression of mutant and WT channels, we used hemagglutinin (HA)-tagged constructs and a chemiluminescence-based technique as described earlier [Estevez et al., 2004; Macias et al., 2007]. The HA epitope was introduced between helices L and M after the glycosylation site, resulting in the sequence "VKHAGYPYDVPDYADPES" (HA epitope in bold). Briefly, oocytes were placed for 30 min in ND96 (96 mM NaCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 2 mM KCl, 5 mM Hepes, pH 7.4) with 1% bovine serum albumin (BSA) at $4^\circ C$, then incubated for 60 min at $4^\circ C$ with 1 μ g ml $^{-1}$ rat monoclonal anti-HA antibody (3F10, Roche) in 1% BSA-ND96, washed at $4^\circ C$, and incubated with horseradish peroxidase-coupled secondary antibody (goat anti-rat Fab fragments; Jackson ImmunoResearch (Suffolk, UK); in 1% BSA for 30–60 min at $4^\circ C$). Oocytes were washed thoroughly (1% BSA, $4^\circ C$, 60 min) and transferred to ND96 without BSA. Individual oocytes were placed in 50 μ l of Power Signal Elisa solution (Pierce, Dallas, TX, USA). Chemiluminescence was quantified in a Turner TD-20/20 luminometer (Turner BioSystems, Sunnyvale, CA).

Cell Culture and Transfection

To counterstain the plasma membrane, cells were incubated at $37^\circ C$ for 10 min using wheat germ agglutinin (WGA) Alexa Fluor 488 conjugate (Invitrogen - Life Technologies) in Hank's balanced salt solution (HBSS) (Gibco - Life Technologies,

Milan, Italy) at 5 µg/ml. The membrane was visualized by excitation at 488 nm. In order to quantify WT CLC-1 and p.Q412P expression, they were alternatively C-terminally tagged with mCherry. mCherry fluorescence was exciting at 535 nm. Images were acquired using an iMIC microscope (TILL Photonics, Munich, Germany) interfaced with TILL Photonics Live Acquisition software using a constant exposition of 20 msec for all images. Data analysis was performed using self-written software (Visual C++, Microsoft). For the analysis, the membrane was manually marked with a region of interest (ROI) using the WGA label. A ring with thickness of ± 3 pixels was then created automatically around the ROI by the software and the mean mCherry fluorescence was calculated within the ring region resulting in the "Membrane fluorescence". The relative membrane fluorescence was calculated by dividing the membrane fluorescence by the total cell fluorescence (as shown in Supp. Fig. S4). Data analysis was finished using the SigmaPlot program (Systat Software, Inc.).

Results

Clinical Results

We clinically investigated four individuals from four unrelated families motivated by the fact that they had been diagnosed as DM1 patients, but molecular diagnosis for DM1 was negative (data not shown). In addition, we evaluated the daughter of one of the probands as she started to present muscle problems. All patients had clear symptoms in agreement with myotonia, except the proband in family 3 and the daughter of the proband in family 4. Table 1 summarizes detailed clinical manifestations found in all five individuals. After performing the clinical re-evaluation and carrying out a deep study of available data from clinical records, patients were diagnosed as: (1) Thomsen disease: probands in families 1, 2, and 4; (2) undiagnosed but with a myotonia-like disorder: proband in family 3; and (3) Myopathy: daughter of the proband in family 4.

Molecular Results

Sequencing and restriction analysis confirmed the presence of four missense mutations in the CLCN1 gene (including one unreported new mutation) (Supp. Fig. S1 and Supp. Table S1) in the four probands included in this study.

After clinical re-evaluation and review of clinical records, we found that the proband in family 1 (F1-CR3; Table 1) had a clinical picture more compatible with the presence of a dominant CLCN1 mutation, but the family history suggested the presence of a recessive mutation (Fig. 1A). We found a C-to-G base change in exon 4, which results in a substitution of phenylalanine for leucine at codon position 167 (p.F167L) (Supp. Fig. S1 and Supp. Table S1). We did not find any other CLCN1 mutation in this proband. This mutation was previously reported both in families with Becker myotonia [George et al., 1994; Modoni et al., 2011] and in Thomsen myotonia families [Fialho et al., 2007]. The affected proband (F1-CR3; Fig. 1A), his unaffected mother (F1-CR2; Fig. 1A) and his unaffected daughter (F1-CR4; Fig. 1A) (according to family information) were heterozygous for this mutation. The proband's father (F1-CR1; Fig. 1A) showed two WT alleles for this genetic variant locus. The proband's daughter was asymptomatic at the moment of blood sampling (11 years old). The proband has 10 siblings, two of which are symptomatic (according to family information) (Fig. 1A). A detailed clinical evaluation in the proband's mother and daughter would be useful to confirm the inheritance pattern of this mutation, as this mutation has been shown to be

present as dominant and recessive, but no further clinical information is currently available in this family.

In family 2 (Fig. 1B), deep retrospective analysis of clinical records (F2-CR5; Table 1) suggested that, as in the above described proband (F1-CR3), the patient had a clinical picture (F2-CR5; Table 1) compatible with the presence of a dominant CLCN1 mutation. However, the screening for CLCN1 mutations led us to the identification of two different recessive mutations in patient F2-CR5: in exon 3, we found a C-to-T base change resulting in the p.R105C amino acid change (p.R105C); in exon 4, we found the same mutation as in family 1 (Supp. Fig. S1 and Supp. Table S1, Fig. 1B). Both mutations were already reported in Becker myotonia families [George et al., 1994; Meyer-Kleine et al., 1995], and they have also been found in combination in some Italian families [Modoni et al., 2011; Brugnoli et al., 2013]. Additionally, the family history suggested a recessive inheritance, making this proband atypical for Thomsen phenotype, which is different from the one reported in Modoni et al. (2011). The proband's sister (F2-CR6; Fig. 1B) also harbored both mutations; however, no clinical information was available for this subject, or for other relatives of this proband.

According to clinical information (retrospective analysis from clinical records), proband F3-CR10 in family 3 (Fig. 1C) might be affected with an inflammatory myopathy such as polymyositis, but showed small myotonic discharges. In this proband, we found an A-to-G base change in exon 4 that results in a substitution of glutamine for arginine at codon position 154 (p.Q154R) (Supp. Fig. S1 and Supp. Table S1). The affected proband was heterozygous for this mutation. This genetic variant is likely to be a new variant in CLCN1 gene as no relevant information was found in the databases (NCBI, Ensembl), with just the Rs appearing (Rs111482384:A>G). As this might be the mutation responsible for the clinical picture, we screened this mutation in 100 unrelated controls to determine if this genetic variant was polymorphic in the Costa Rican population. However, we did not find this variant in 200 unrelated chromosomes (data not shown). This new variant has been submitted to the LOVD database (<http://www.lovd.nl/CLCN1>). We did not find any other CLCN1 mutation in this proband. A comparison of CIC-1 channel sequences of various species shows that the glutamine at codon position 154 is not highly conserved (Supp. Fig. S2). We additionally screened some of the proband's relatives. Her mother (F3-CR8; Fig. 1C) and her niece (F3-CR12; Fig. 1C) were heterozygous for the mutation but with no known clinical evidence of myotonia. The other relatives tested in this family did not carry this genetic variant (F3-CR7, F3-CR9, and F3-CR11; Fig. 1C). No further clinical information is available for this family.

Proband F4-CR14 (Fig. 1D) in family 4 was diagnosed with Thomsen disease based on clinical evaluation and review of clinical records (F4-CR14; Table 1). In this proband, we found a mutation previously reported by us in a Becker myotonia family (Morales et al., 2008): an A-to-C base change in exon 11, which resulted in a substitution of glutamine for proline at codon position 412 (p.Q412P) (Supp. Fig. S1 and Supp. Table S1). Family history suggested a recessive inheritance but we did not find any other CLCN1 mutation in this proband. F4-CR14 has two unaffected children (F4-CR17 and F4-CR18; Fig. 1D), one of which (F4-CR17) was heterozygous for this mutation. His mother and two siblings were asymptomatic (self-reported) carriers for this mutation. EMG of F4-CR17 showed a myopathic profile but not the characteristic myotonic discharges (F4-CR17; Table 1). No further clinical information is available for this family.

Biophysical Analysis

In order to understand the functional consequence of the above described CLCN1 mutations and their role in myotonia we first characterized the biophysical properties of the p.Q154R, p.R105C, p.F167L and p.Q412P CIC-1 channels individually. When expressed in *Xenopus* oocytes, no obvious differences regarding kinetics and current magnitude were observed for p.R105C, p.Q154R, and p.F167L compared to WT using the two-electrode voltage-clamp method (Fig. 2A–D and F). A detailed quantitative analysis of current properties was performed using inside-out patch clamp experiments as described below. In contrast, mutant p.Q412P showed only very small macroscopic currents (Fig. 2E and F), with properties similar to those of WT (see below). To investigate in a quantitative manner the biophysical properties of these mutants, we performed inside out patch clamp experiments and determined separately the fast and common gate properties using appropriate pulse protocols [Accardi and Pusch, 2000] (Fig. 3A and B). None of the mutations exhibited significant alterations of gating parameters for both gates (Fig. 3C–H). Also kinetic analysis of current relaxations at –140 mV did not reveal any significant differences (Supp. Fig. S3). Furthermore, noise analysis was used to estimate the single channel conductance [Pusch et al., 1994; Saviane et al., 1999]. Again, none of the mutants significantly affected this parameter (Fig. 3I).

To investigate the mechanism underlying the reduction of the macroscopic conductance of mutant p.Q412P, we assayed the surface membrane expression of HA-tagged constructs using a chemiluminescence-based technique [Estevez et al., 2004; Macias et al., 2007]. As shown in Figure 4A, mutant p.Q412P exhibits a dramatically reduced surface expression compared to WT channels, in full agreement with the reduced macroscopic conductance but unchanged single channel conductance or open probability. Thus, mutant p.Q412P either is inefficiently produced and targeted to the plasma membrane or it is more rapidly degraded.

Because the p.Q412P mutation was found in the proband in heterozygous status, without other mutations in CLCN1 identified by the methods used, we next asked if the mutant shows a dominant effect in heterologous expression systems. To mimic the heterozygous status of patients we coinjected WT and mutant RNA in a 1:1 ratio and compared the macroscopic conductance with that seen in oocytes which had been injected with the half amount of WT RNA, and using as a second control a coinjection with RNA coding for the known recessive mutation p.M485V [Wollnik et al., 1997]. However, no clear dominant negative effect on WT CIC-1 could be detected (Fig. 4B). To test whether p.Q412P may display dominant negative effects on its WT counterpart in a hetero-dimer configuration in the plasma membrane, we additionally coexpressed mutant and WT at molar ratios of 3:1 and 10:1. However, even with this large excess of mutant RNA, no dominant negative effect could be observed (Fig. 4B). One possibility to explain the lack of dominance is that during its biogenesis, p.Q412P is unable to interact with WT CIC-1, and thus is unable to reduce the surface expression of the dimeric complex. Alternatively, WT CIC-1 could lead to a partial rescue of the mutant, that is, heterodimeric mutant/WT complexes might be correctly targeted into the plasma membrane to a certain extent.

To test this latter hypothesis, we individually C-terminally tagged WT CIC-1 or the p.Q412P mutant with the red fluorescent protein mCherry and tested their overall and the plasma membrane expression in transfected HEK293 cells. These constructs

retained full functionality (data not shown). In these experiments, the plasma membrane was identified by Alexa-Fluor labeled WGA.

Although WT CIC-1-mCherry transfected alone was clearly detected in the cytoplasm and in the plasma membrane (Fig. 4C and G), Q412P-mCherry fluorescence was dramatically reduced (Fig. 4D and G) in agreement with the surface expression analysis in oocytes. Furthermore, coexpressing unlabeled p.Q412P with CIC-1-mCherry did not reduce WT plasma membrane mCherry fluorescence significantly, in agreement with the lack of dominance in the electrophysiological analysis (Fig. 4E and G). Finally, coexpressing unlabeled CIC-1 with Q412P-mCherry did not lead to a significant increase of mutant protein in the plasma membrane (Fig. 4F), showing that WT subunits were not able to “rescue” the mutant. Interestingly, the relative membrane localization of p.Q412P was not different compared to WT (Supp. Fig. S4). Thus, from these experiments it appears that the p.Q412P mutant is unable to interact with WT CIC-1, most likely because it is degraded before having a significant chance to dimerize with other subunits. However, even though the mutation is not localized at the dimer interface, there is still the possibility that it impairs the formation of heterodimers, because it specifically influences the dimerization process.

Discussion

In the present manuscript, we identified and functionally characterized genetic mutations in the CLCN1 gene in four Costa Rican families that had previously been diagnosed with DM1 but that resulted negative for the DM1 mutation. The mutations found in families 1 (p.F167L), 2 (p.F167L and p.R105C), and 4 (p.Q412P) are well-known mutations found in other families with MC [George et al., 1994; Meyer-Kleine et al., 1995; Zhang et al., 2000; Fialho et al., 2007; Morales et al., 2008; Modoni et al., 2011; Brugnoli et al., 2013; Desaphy et al., 2013]. We can thus safely conclude that the affected members of these families should be diagnosed as having MC and not DM1. Although the p.F167L mutation (found in F1-CR3) has been found cosegregating with myotonic dystrophy type 2 (DM2) [Cardani et al., 2012], we did not perform the molecular diagnosis of DM2 in proband F1-CR3 because the clinical picture of the patient and the inheritance pattern (dominant in DM2) in the family suggested that the patient was affected with MC instead of DM2. There has been also one report describing the copresence of CLCN1 and SCN4A mutations in the same patient [Furby et al., 2014]. Thus, it might be possible that proband F1-CR3 carries a mutation in the SCN4A gene. However, this is unlikely because the inheritance pattern of the disease in this family is recessive (SCN4A mutations are dominant) and because the clinical picture is more suggestive of MC than a sodium channelopathy. Therefore, we did not look for mutations in the SCN4A gene in this proband.

Proband F2-CR5 shows a clinical Thomsen phenotype but carries two CLCN1 mutations (p.R105C and p.F167L). As for proband F1-CR3, we did not test for cosegregation of SCN4A mutations in proband F2-CR5 because the clinical picture and the recessive inheritance pattern in the family are clearly suggestive of MC rather than a sodium channelopathy. Recently, Brugnoli et al. (2013) reported two brothers with the p.F167L and p.R105C mutations in association with a third CLCN1 mutation (Q812X). However, we did not find evidence of a third mutation in the CLCN1 gene in proband F2-CR5. An additional mutation in these probands might help to explain their atypical MC phenotypes.

In contrast, we cannot conclude that the mutation p.Q154R found in family 3 is indeed

underlying the disease phenotype. First, the Gln residue at position 154 is not conserved in the CIC-1 sequence among different mammalian species. For example, the chimp CIC-1 sequence bears an Arg residue at position 154 (Supp. Fig. S2). In addition, residue 154 is localized in the extracellular loop between helices B and C, which is poorly conserved among CLC channels. Second, other family members have the same variations with no clinical manifestations. Third, the p.Q154R mutation is considered a tolerated/benign variant in the Ensembl database (http://www.ensembl.org/Homo_sapiens/Variation/Mappings?db=core;r=7:143320892-143321892;v=rs111482384;vdb=variation;vf=18911405) based on the application of the SIFT (<http://sift.jcvi.org>) and the PolyPhen (<http://genetics.bwh.harvard.edu/pph2/>) prediction programs that are routinely applied in the Ensembl database. The SIFT prediction is based on the degree of conservation of amino acid residues in sequence alignments [Kumar et al., 2009] whereas the PolyPhen prediction tool uses additional physical parameters of substituted amino acids [Adzhubei et al., 2010]. Finally, our functional analysis in *Xenopus* oocytes could not detect any functional difference compared with WT CIC-1 regarding macroscopic conductance, voltage dependent gating and single channel conductance.

Several previous studies could not detect functional differences for mutations p.F167L and p.R105C [Zhang et al., 2000; Fialho et al., 2007; Desaphy et al., 2013]. Here, we studied in even more biophysical detail these mutations, separating the fast and slow gating components [Saviane et al., 1999; Accardi and Pusch, 2000] and determining their single channel conductance [Pusch et al., 1994]. However, even these detailed measurements did not reveal any functional difference of the disease causing mutations p.F167L and p.R105C compared with WT. In contrast, for p.F167L, Lucchiari et al. (2013) have found a very small increase of the open probability of the common gate at negative voltages. This different result may be caused by different experimental conditions related to the recording conditions or the different intracellular chloride concentration employed. The two mutations are also not close to exon/intron boundaries rendering unlikely an involvement in splicing. Thus, for these mutations, and several other missense mutations [Pusch, 2002; Lossin and George, 2008], there must exist other molecular mechanism underlying myotonia in these patients. These might be at the transcriptional, translational, or at the protein level. This is an important problem to address in the future using models that are closer to the *in vivo* situation.

Although the p.F167L mutation has been reported as recessive in most families with a clear Becker myotonia phenotype [George et al., 1994; Modoni et al., 2011], we found it in a patient with a clinical picture more compatible with Thomsen myotonia but with a family history that suggest a recessive inheritance pattern (Fig. 1A). However, the proband's mother, who is a carrier of this mutation, considers herself asymptomatic, but no detailed clinical data are available regarding this subject. It is still possible that she presents some milder symptoms that are unnoticeable to her. In addition, in this study just one of the proband's children was clinically tested in this project, and although she was not affected at the moment of this study, she is not yet beyond the age of onset on MC [Becker, 1977]. Thus, detailed clinical evaluation is required for these two subjects to rule out the possibility that the mutation is presenting as a dominant mutation in family one, similar to what described Fialho et al. (2007). Thus, like several other mutations [Kubisch et al., 1998; Plassart-Schiess et al., 1998; Colding-Jorgensen, 2005], p.F167L may show incomplete penetrance in most families, but can be dominant in some families. However, the evidence so far indicates that it is more likely that this is a recessive mutation. If this is the case, the proband should carry

a second CLCN1 gene defect, like a splice-site mutation in heterozygosity with p.F167L, as found recently [Ulzi et al., 2014] or exon deletion/duplications [Raja Rayan et al., 2012; Ulzi et al., 2014]. However, we did not find any putative splice-site mutation and multiplex ligation-dependent probe amplification (MLPA) assay excluded exon deletion/duplications in CLCN1 in this proband (data not shown). It might also be possible that this proband carries a mutation in another gene, such as SCN4A or CAV3. The CAV3 gene encodes a major component of caveolae in skeletal and cardiac muscle and mutations in this gene have been associated with limb-girdle muscular dystrophy 1C, distal myopathy, rippling muscle disease, isolated hyperCKemia, cardiomyopathy and recently, with myotonia [Milone et al., 2012]. Alternatively, mutations in other unidentified myotonia-associated genes cannot be excluded, as a mutation in a different gene in the same patient could modulate a muscle phenotype [Furby et al., 2014]. Another possibility is that a difference in allelic expression underlies the myotonic phenotype in the affected patient [Duno et al., 2004] or that this mutation also presents as dominant with low penetrance.

The most interesting and novel findings regard the p.Q412P mutation. This mutation has been previously identified in a clearly recessive inheritance pattern in a patient with clinically Becker type myotonia [Morales et al., 2008]. The clinical picture of the proband in family four (Fig. 1D) in this study is more likely to suggest that the patient is affected by Thomsen disease (F4-CR14; Table 1) with no second mutation identified in this heterozygous patient, suggesting a dominance of the p.Q412P mutation. However his other relatives, who underwent DNA testing carrying the same p.Q412P mutation, showed no myotonia, and the family history suggested a recessive inheritance. Like in family one, we could not detect a second mutation in the CLCN1 gene in this proband and he did not carry exon deletion/duplications in the CLCN1 gene (data not shown). Thus, as for the proband in family one, it might be possible that this proband also carries a mutation in other gene.

The p.Q412P mutant showed an interesting phenotype in heterologous expression systems. The overall conductance induced in *Xenopus* oocytes was dramatically reduced but not completely abolished. Surprisingly, the biophysical properties, including voltage dependence of fast and slow gate and the single channel conductance, of the remaining current carried by homodimeric p.Q412P channels were indistinguishable from those of WT. These electro- physiological results suggest that p.Q412P exhibits a reduced surface plasma membrane expression compared to WT. This conclusion was indeed confirmed using an antibody-based chemiluminescence assay. Furthermore, in HEK293 cells transfected with an mCherry fusion protein the fluorescence of the mutant was dramatically reduced, suggesting a severe folding or maturation defect. The fact that the biophysical properties of the conducting p.Q412P channels are identical to those of WT demonstrates, however, that in principle the mutant is able to form fully functional dimeric channels, and that it does not significantly affect the structure of the fully assembled channel. Thus, it seemed plausible that the mutant might confer a dominant negative effect on WT CIC-1 by heterooligomerization with WT subunits. However, we could not detect any significant dominant negative effect in electrophysiological experiments in *Xenopus* oocytes. In addition, cotransfection of WT CIC-1-mCherry with unlabeled p.Q412P did not reduce the plasma membrane fluorescence or induce an increase of intracellular fluorescence, suggesting that it is not able to interact with WT. However, an alternative explanation for this lack of negative dominance could be that mutated p.Q412P subunits are rescued and expressed in the plasma membrane by heterooligomerization with WT

subunits. In this case, mCherry-tagged mutant protein should be dragged to the plasma membrane when coexpressed with WT. However, we excluded this possibility by showing that cotransfection of unlabeled WT CIC-1 with Q412P-mCherry did not induce any significant plasma membrane fluorescence.

From these various pieces of evidence, we conclude that the mutant p.Q412P induces a severe folding defect that leads to its degradation before the mutated subunit is able to dimerize in a significant proportion with WT subunits. As the protein appears to be stable with WT properties once inserted in the plasma membrane, it can be speculated that the structural change induced by the mutation is only important during the biogenesis in the endoplasmic reticulum, leading to early degradation. However we cannot fully exclude the possibility that the mutation impairs the formation of heterodimers. These properties are in full agreement with a recessive mode of inheritance, which is largely compatible with the genetic data. Interestingly, several myotonia-causing mutations are located in the vicinity of p.Q412 in the extracellularly exposed K-L loop, including p.F413C [Koch et al., 1992] (one of the most common MC mutations found [Lossin and George, 2008], p.P408A [Fialho et al., 2007], p.A415V [Mailander et al., 1996], and p.E417G [Trip et al., 2008]). Among these, p.F413C has been shown to impair endoplasmic reticulum export [Papponen et al., 2008], whereas p.E417G expressed at similar levels as WT CIC-1 [Zhang et al., 2006].

In conclusion, our study confirms the presence of the myotonia causing CLCN1 mutations p.F167L and p.R105C in the Costa Rican population. The molecular disease mechanism for these two mutations remains obscure. No firm conclusion can yet be drawn for the p.Q154R mutation, which may be a benign variant. The mutation p.Q412P in the extracellular K-L loop induces a severe folding defect disabling to a large extent dimerization, explaining the lack of dominance associated with the mutation in patients and heterologous expression systems. The mutation, however, does not significantly affect the structure and function of the protein if the folding defect can be overcome, as happens to a minority of channels in the oocyte expression system. This aspect is of particular importance regarding possible therapies targeted at improved folding that might restore full functionality.

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Figure legends

Figure 1. Pedigrees of the four families segregating CLCN1 mutations.

A: Family 1 carries the p.F167L mutation in exon 4. B: Family 2 carries p.R105C and p.F167L mutations in exons 3 and 4, respectively. C: Family 3 carries the p.Q154R mutation in exon 4. D: Family 4 carries the p.Q412P mutation in exon 11. There is no complete clinical information and genomic DNA available for family members with no code.

*Figure 2. Currents mediated by WT and the indicated mutants measured by two-electrode voltage clamp in *Xenopus* oocytes.*

A–E: Typical current traces evoked by a protocol applied using a prepulse to +60 mV for 100 ms followed by steps from –140 to +80 mV in 20 mV increments and a final tail pulse to –100 mV. F: Slope conductance estimated at the reversal potential from steady-state current voltage plots.

Figure 3. Biophysical properties of mutants and WT.

Panels A and B show the voltage clamp protocols to separate the fast and the slow gate properties (see Methods). Typical current traces from inside out patches with these protocols are shown for WT (C and D) and mutant p.Q412P (E and F) (note the different current scale in E and F). The voltage of half maximal activation obtained from these protocols using the Boltzmann analysis (see Methods) is shown in E and F. The single channel current at –140 mV obtained from noise analysis is shown in I for WT and mutants.

Figure 4. Expression and interaction analysis of mutant p.Q412P with WT CIC-1.

A: Chemiluminescence assay for surface membrane expression shows a reduced expression of p.Q412P compared with WT CIC-1 (water-injected oocytes: n = 19; WT CIC-1: n = 15; p.Q412P: n = 17). B: Coexpression of WT and mutant p.Q412P in oocytes in a ratio of 1:1 to mimic the heterozygous status of patients and coexpression in ratio of 1:3 and 1:10, maintaining cRNA WT amount of 2.5 ng, to observe the effect of increased mutant protein amount on the WT. The macroscopic conductance was measured 2 days after injection. The recessive p.M485V mutant [Wollnik et al., 1997] served as a control of a not dominant-negative effect (number of oocytes: NI [noninjected]: n = 9; WT 2.5 ng: n = 10; WT 5 ng: n = 12; p.Q412P 5 ng: n = 11; p.Q412P/WT 1:1: n = 11; p.Q412P/WT 3:1: n = 12; p.Q412P/WT 10:1: n = 11; p.M485V 5 ng: n = 11; p.M485V/WT 1:1: n = 12; p.M485V/WT 3:1: n = 9; p.M485V/WT 10:1: n = 11. Error bars: SD; values for p.Q412P/WT coexpressions are not statistically different from the “WT 2.5 ng” value ($P > 0.05$) and not significantly different from the respective M485V/WT values (except for the 10:1 ratios for which [$P < 0.001$]; similar results were obtained in a total of three batches of oocytes). C–F: mCherry-tagged DNA of WT CIC-1 (C) or p.Q412P (D) was transfected in HEK293 cells alone (C and D) or with untagged p.Q412P (E) or WT CIC-1 (F), respectively. All images (C–F) have the same exposition time (20 ms) and background subtraction. The plasma membrane was identified with Alexa labeled WGA (see Methods) which was excited at 488 nm (left pictures in panels C–F). Since p.Q412P expresses only very little protein, the right images in D and F are displayed with a ~17-fold larger contrast compared to those of C and E (gray scale ranges from pixel image values of 280 to 4000 in C and E, and from 280 to 500 in D and F). Scale bar, 15 μ m. G: Membrane fluorescence analysis of several images as shown C–F. For each cell, the membrane was defined by the WGA label and the mCherry fluorescence was averaged in a ring of ± 3 pixels around the

membrane. Note the very small membrane fluorescence of p.Q412P-mCherry and p.Q412P-mCherry/WT compared with WT-mCherry (error bars: SEM; ***: $P < 0.001$, n.s.: not significant ($P > 0.05$), Student's t-test; WT-mCherry: $n = 22$; p.Q412P/WT-mCherry: $n = 33$; p.Q412P-mCherry: $n = 44$; p.Q412P-mCherry/WT: $n = 32$).